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DRUG DISCOVERY

In vitro and *In vivo* Antiproliferative activity of extracts and fractions of leaves and stem from *Tabebuia hypoleuca* (C. Wright) Urb.

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Background: ***Tabebuia hypoleuca*** **(C. Wright) Urb. Is Cuban endemic plant without preview study as antitumor, however these genera had been reported with several pharmacological activities.** ***Purpose:*** **The aim of this study was to investigate *in vitro* and *in vivo* antiproliferative activity of extracts and fractions of leaves and stems from *Tabebuia hypoleuca* (C. Wright) Urb and study the chemical composition in the major fraction of its stems (TT2).** ***Methods:*** **In vitro antiproliferative activity was evaluated in 8 human cancer cell lines: melanoma (UACC-62), breast (MCF7), kidney (786–0), lung (NCI-H460), prostate (PC-3), ovary (OVCAR-3), colon (HT29), and leukemia (K-562) and one no tumor line (HaCat). Total growth inhibition (TGI) values were chosen to measure antiproliferative activity. In vivo studies were carried out with Ehrlich solid tumor on Swiss mice and Hollow fiber assay with MCF7.*** ***T. hypoleuca*** **leaves ethyl acetate crude extract (THA), semi pure compounds (TH 3-6 and TT2 -11) and positive control, were used.** ***Results:*** **Among the cell lines evaluated, THA, THA (3-6), TT2 demonstrated better *in vitro* antiproliferative activity towards kidney, glioma, breast, lung, prostate, ovary, colorectal and leukemia cancer cell lines. The intraperitoneal administration of THA, THA 3-6 significantly inhibited tumor progression in comparison to control (saline) and shown reduction in the inhibition of the proliferation of MFC-7 human tumor cell at the doses testing. TT2 (11) isolated of stems was a mixing of hopenone b (major proportion), lupenone andtaraxerone.** ***Conclusions:*** **Therefore, this study showed the presence of different pentacyclic triterpenes in *T. hypoleuca* and it have promising anticancer activity. Further studies will be undertaken to determine the mechanism of action and toxicity of these compounds.**

INTRODUCTION

Cancer is still a serious clinical problem and has a significant social and economic impact on the human health care system. Despite modern advancements in diagnosis, prevention and therapy, the disease still affects millions of patients worldwide, reduces their quality of life and one of the leading causes of death in the world (Melike Demir Doğan et al. 2018; Khalid Obiad Mohsin Almohammadawi et al. 2018). Accord World Health Organization, WHO, each year appears near ten millions of new patients of cancer responsible of six millions of death. WHO suggest 27 millions of cancer patients in 2030 with the major incidence of melanoma, prostate, lung, stomach, colon and rectum in men and for women more incidence of skin, mama, uterus, thyroid glands, colon and rectum (IARC, 2011).

Natural products including plants, microorganisms and marines provide rich resources for anticancer drug discovery (Udayakumar Mani, 2013; Sivaprakasam Ramya et al. 2018) and some time these products are not use as they were isolated but they are inspiration at the development of new potential agents (Cragg and Newman, 2009). As the different components in a herb may have synergistic activities or buffering toxic effects, mixtures or extracts of herbs might have more therapeutic or preventive activity than alone (Vickers, 2002; Yeung-Leung et al., 2005; Suresh Babu et al. 2016; Neil K Agarwal and Shashi K Agarwal, 2016).

Tabebuia spp. (Bignoniaceae) includes approximately100 species, known as strictly woody, found in tropical rain forest areas throughout Central and South America (Olmstead et al., 2009). Species of the genus *Tabebuia* have been traditionally used to treat syphilis, malaria, cutaneous infections, stomach disorders, cancer, inflammation, pain, bacterial and fungal infections, anxiety, poor memory, irritability, depression, and others (Cragg et al., 2014; Sadananda et al., 2011; Gomez et al., 2012; Franco, 2013; Ferreira et al., 2014). *Tabebuia*

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hypoleuca (C. Wright ex Sauvalle) Urb., commonly known as "Roble macho", is an endemic species inCuba, native to the Sierra Maestra. We have previously reported the anti-inflammatory, antinociceptive and antipyretic activities of the methanol extract from *T. hypoleuca* stems (Regalado et al., 2015; 2017; 2017a).

Despite several studies related to the *Tabebuia* genus, no evidence of *in vitro* or *in vivo* anticancer activity of *Tabebuia hypoleuca* had been reported previously.

The development of experimental models has contributed to the study of antineoplasic compounds and to the understanding of their mechanism of action. Solid tumors are structures resembling organs in their complexity and heterogeneity, with a microenvironment formed by tumor and stroma cells which are embedded in the extracellular matrix and in the presence of a vascular network. These parameters often contribute to tumor resistance to chemotherapy due to irregular distribution of drugs inside the tumor matrix. Therefore, the development of in vivo experimental models to complement *in vitro* drug screening is necessary due to the limitations inherent to cell cultures to predict the behavior of solid tumors to chemotherapy (Trédan et al., 2007).

There are a number of experimental models based on laboratory animals including the Ehrlich solid tumor, derived from a mouse breast adenocarcinoma which is an aggressive and fast growing carcinoma able to develop both in the ascitic or solid form depending whether inoculated intraperitoneously or subcutaneously, respectively (Nascimento et al., 2006).

Transplanted tumors afford an advantage due to the previous knowledge of the amount and initial features of the tumor cells inoculated and to the fast development of the neoplasia, thus reducing the duration of the study (Stewart, 1959).

The aim of this study was to investigate *in vitro* and *in vivo* antiproliferative activity of extracts and fractions of leaves and stems from *T. hypoleuca* and study the chemical composition in the major fraction of its stems.

MATERIAL AND METHODS

General experimental procedures

Column chromatography (CC): silica gel 60 (70- 325mesh, Merck®, 2 × 50 cm). TLC (thin-layer chromatography): precoated plates (Merck®), UV detection, and anisaldehyde solution.

Plant material, extraction and fractionation

T. hypoleuca were collected at the National Botanical Garden (JBN), Havana Province, Cuba. The identification of the plant was confirmed by Dr. Eldis R. Becquer and a sample was deposited in the herbarium of the experimental station with the number HFC-88204.

Its leaves and stem were separated and dried at room temperature. Solid-liquid successively extraction in Soxhlet increasing the polarity of the solvent were used with n-hexane, ethyl acetate and methanol (Merck ®) by leaves and extraction with methanol by stems. Each extract were filtered and concentrated using rotary evaporation, providing the crude hexanic (THH, 1.15 % yield), ethyl acetate (THA, 2.59 % yield) and methanol (THM, 4.09 % yields) extracts by leaves and crude methanol (TTM, 6, 64 %) extract with stems.

In vitro cytotoxic assay monitored the extracts activity, showing that THA was active. An aliquot (2.5 g) of THA was chromatographed over silica gel (80 g), eluted first with hexane. The eluent polarity was increased by gradients of ethyl acetate and then methanol, providing sixteen fractions (100mL). They were grouped according to the thin-layer chromatography(TLC) profile, visualized with anisaldehyde reagent(50mL acetic acid, 0.5 mL sulfuric acid, and 0.5mL anisaldehyde) followed by heating at 110°C. It was observed a high quantity of solid in fractions elute with n-hexane- ethyl acetate mixture(THA 3 to7).A similar process was make to THM and it were obtain 24 fractions.

Total methanol extract from stems (TTM, 21.9 g) were fractionated using a column with 50 g silica gel 60 (0,063-0,200 mm) 70–230 mesh ASTM; Merck. The column was eluted with solvent increasing in polarity to petroleum ether (apolar) up methanol – water (polar). It was collected six fractions: TT1 (petroleum), TT2 (petroleum – ethyl acetate, 1:1), TT3 (ethyl acetate), TT4 (ethyl acetate – methanol, 1:1), TT5 (methanol) y TT6 (methanol-water). In vitro cytotoxic assay monitored the fractions activity, showing that TT2 was active and the major yielding fraction in the stems. Using column chromatography of TT2 was isolated the compound TT2 (11).

Chromatographic analysis

The GC/MS analysis of compound TT2 (11) was carried out using Agilent technology Chromatograph, HP-6890/5975 system equipped with an HP-5 (30m× 0.25mm×0.25 µm). Temperature program: 110°C (5°C·min−1) – 280°C (60 min), injector 250°C, detector 300°C. Helium was used as a carrier gas (0.7 bar, 1mL ·min−1). The MS were taken at 70 eV. Scanning speed was 0.84 scans s−1, from 40 to 550. Sample volume was 1 µL. Split: 1: 40. The mass spectra were compare with software NIST05 MS Search. This analysis suggested the presence of compounds 1.

In vitro antiproliferative assay

Cell lines

Human tumor cell lines U251 (glioma), MCF-7 (breast),NCI-460 (lung, non-small cells), OVCAR-03 (ovarian), PC-3 (prostate), HT-29 (colon), 786-0 (renal), K-562 (leukemia), NCI-ADR/RES (ovarian expressing phenotype multiple drugs resistance) were obtained from National Cancer Institute/USA and one no tumor cell line HaCaT (immortal keratinocyte from human skin) obtain from Prof. Dr. Ricardo Della Coletta (FOP/UNICAMP).

Cell culture

Stock cultures were grown in medium containing 5 mL RPMI1640 (GIBCO BRL) supplemented with 5% fetal bovine serum (FBS, GIBCO) at 37℃ with 5% CO2. Penicillin: streptomycin (1000 µg/L: 1000 U/L, 1 mL/L) was added to the experimental cultures.

Antiproliferative assay

Cells in 96-well plates (100 µL cells well^{-1}) were exposed to extracts, fractions and isolated compound concentrations in DMSO (Merck)/RPMI(0.25, 2.5, 25, and 250 µgmL^{-1}) at 37ºC, 5% of CO2 in air for48 h. Final DMSO concentration did not affect cell viability. Afterwards cells were fixed with 50% trichloroacetic acid (Merck) and cell proliferation determined by spectrophotometric quantification (540 nm) of cellular protein content using sulforhodamine B assay (Monks et al., 1991).

Using the concentration–response curve for each cell line, the TGI (concentration that produces total growth inhibition or cytostatic effect) and GI50 (concentration that produces 50% growth inhibition) were determined through non-linear regression analysis (Table 1) using software ORIGIN 7.5® (OriginLab Corporation) (Shoemaker, 2006).

Table 1 Antiproliferative activity of doxorubicin, THH, THA, THM and against three human cancer cell lines

	TGI (ug/ml)		
	U251	**MCF-7**	**786-0**
Doxorubicin	0,82	0,39	**0,29**
TT-2	25,9	8,5	**35,5**
TT-3	151,8	139,6	**124,3**
TT-4	>250	166,7	**>250**
TT-5	>250	>250	**>250**
TT-6	>250	>250	**>250**
THA-1	83,1	115,4	**70,5**
THA-2	>250	>250	**>250**
THA-3	52,5	32,5	**65,7**
THA-4	49,1	39,5	**14,3**
THA-(5-6)	68,9	91,1	**65,4**
THA-7	60,2	58,7	**26,7**
THA-8	>250	>250	**>250**
THA-(9-14)	125,0	59,1	**117,7**
THA-(15-16)	>250	>250	**>250**
THH	70,3	60,3	**61,2**
THA	109,8	95,3	**53,7**
THM	>250	>250	**>250**
THM-2	97,1	132,0	**212,8**
THM-3	115,9	54,8	**64,2**
THM-4	174,8	57,9	**89,9**
THM-5	69,9	78,2	**79,6**
THM-6	100,8	77,9	**94,2**
THM-7	>250	184,0	**>250**
THM-8	>250	>250	**>250**
THM-9	>250	>250	**>250**
THM-(10-12)	>250	>250	**>250**
THM-(11'-12')	105,0	61,8	**117,2**
THM-13	>250	>250	**>250**
THM-(15'-18')	>250	>250	**>250**
THM-19'	>250	>250	**>250**
THM-(20'-21')	>250	>250	**>250**
THM-(22'-24')	**>250**	**>250**	**>250**

TGI values represent the necessary concentration (µg/mL) for total inhibition of cancer cells proliferation. Values were determined through nonlinear regression analysis using the ORIGIN 7.5® (OriginLab Corporation). Dose range tested 0.25 to 250 µg/ml. Doxorubicin (positive control).

The assay results for the samples screened were analyze by two different classification, one according to National Cancer Institute: GI50 < 30 µg/mL over tumor cells were positive (Oskoueian et al., 2011) (GI50 ≤30 µg mL^{-1}, potent activity; 31<GI50 < 60 µg mL^{-1}, moderate activity and 61< GI50 <99µg mL–1, weak activity). In addition, the second, according to Fouche et al., 2008: inactive (TGI > 50 µg/mL), weak activity (15 µg/mL < TGI < 50 µg/mL), moderate activity (6.25 µg/mL < TGI < 15 µg/mL), and potent activity (TGI < 6.25 µg/mL). Extracts and fractions were selected according these two categories for further *in vivo* testing for anticancer activity.

In vivo assays

Animals

Female or male Swiss mice aged 8–10 weeks and weighing 20–35 g were used *in vivo* assays. The animals were obtained from CEMIB-UNICAMP were maintained in a room with controlled temperature 25 ± 2℃ for 12h light/dark cycle, with free access to food and water. All procedures were accordance with the principles and guidelines adopted by the institutional Committee for Ethics in Animal Research, at the State University of Campinas (CEEA, UNICAMP, protocol1 and 2, July and August 2013).

Acute toxicity

Swiss mice were treated intraperitoneal (i.p.) with ethyl acetate extract from leaves (THA, 50 and 100 mg/kg). Groups were observed during4 h and then daily for 14 days. The following parameters were evaluated: general toxicity signals like body weight loss, locomotion, behavior (agitation, lethargy), respiration, salivation, tearing eyes, cyanosis, and mortality (Lapa et al., 2003; 22; Litchfield and Wilcoxson, 1949).

Ehrlich solid tumor (footpad)

THA was evaluate *in vivo* onan Ehrlich solid tumor assay. Ehrlich tumor cells, mammary adenocarcinoma were maintained in the ascites form by peritoneal passages in mice by weekly transplantation of 5×10^5 tumor cells. For testing, cells were prepared at a density of 2.5×10^6 cells/60 µL/animal [23].Ehrlich tumor cells (2.5×10^6) were implanted on the right footpad and the animals were treated with the samples, i.p. every72 h. The animals (6 female mice/group) were dividing into groups: negative control group (saline), positive control group (5-fluorouracil, 25 mg/mL; Europharma) at dose of 20mg/kg and sample treated groups, THA. THA was administering at doses of 50, 75, and 100 mg/kg. To determine the solid tumor growth, footpad volume was measure every 3 days, using a digital caliper, until the fifteenth day, approximately, when the animals were sacrifice. Anticancer activity was assessing by comparing the tumor volumes using the equation: difference between the volumes of the paw with tumor (edema) respect to healthy paw. Finally, both paws were remove and weighed, providing the relative tumor weight, calculated as the difference between the weights of the paw with tumor and of the healthy paw. The relative weight of organs was evaluate as indicator of toxic effect of the treatments.

Ehrlich solid tumor (back)

In this experiment was followed the same methodology describe above. Ehrlich tumor cells (1×10^7) were then inject subcutaneously in their back (60 µl) where the tumors were develop in a single and solid form in seven days. The animals were treated with the samples every 72 h during 20 days when they were sacrificed (i.p., considering day seven as initial time).The animals (8 male mice/group) were divide into groups: negative control group (saline), positive control group (5-fluorouracil, 25 mg/mL; Europharma) at dose of 20mg/kg and sample treated groups, THA or fraction THA (3-6). HA was administered at doses of 100 and 150 mg/kg; THA (3-6) was administrated at dose of 25 and 50 mg/Kg. The tumors were weighting. Then, it were evaluated the relative organs´ weight and blood biochemistry as toxicity indicator.

Statistical analysis

Results were expressed as the mean ± standard deviation. All results were submitted to one way analysis of variance (ANOVA),considering as critical level p <0.05 to evaluate significant difference between the control and treated groups, followed by Dunnet´s Multiple Comparison Test, using Graphpad Prism, Version 5.

Hollow fiber assays with MCF-7 (breast adenocarcinoma)

In this study were used MCF-7 human cancer cell lines in immune-competent BALB/c mice, female weighing 18- 22 g. Cells was culture in normal condition (medium RPMI + FBS 5%, streptomycin/penicillin, 37℃). The cells were harvested by a standard trypsinizatation procedure and resuspended at the desired cell density (1 x 10^5cells/fiber, in 160 µl of culture medium). The cell suspension was flushed into the hollow fibers, where after they were heat-sealed and cut at 2 cm intervals. The fibers were incubated in culture medium in six-well plates 24 h prior to

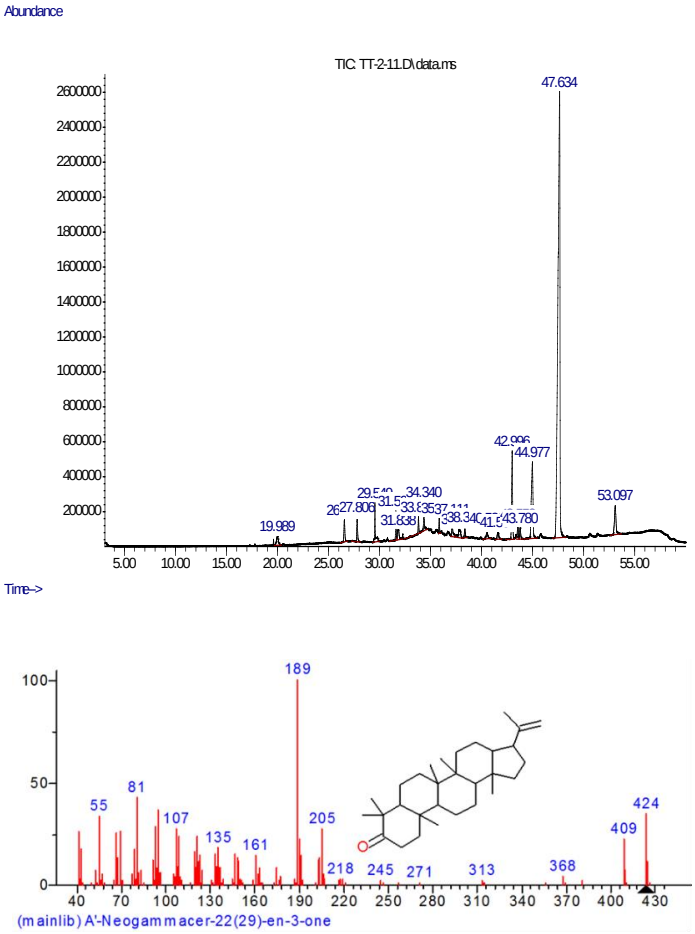


Figure 1 Chromatography profile and Mass Spectrum-structure obtain byGC/MS of TT2-11 obtain from TT2 fraction (petroleum- ethyl acetate) of Tabebuia hypoleuca stems

surgical implantation inimmune-competent6- to 8-week-old female pure strain BALB/c mice. Three fibers were implanted s.c. with MCF- 7 (breast adenocarcinoma) on the mice flank. The mice were anesthetized by 30 mg/Kg de pentobarbital sodium (day 0), and the incisions were closed using a stapling device. Separate *in vitro* control fibers were also prepared, and were incubated in medium during the experiment (7 days) as control for evaluate the quantity of cells/fibers in the moment of animal´s implanted and cell growing was evaluated by MTT method (Decker *et al*, 2004).

Treatments (mice, n=5/ group) were made daily (6 administration), i.p., with negative control (vehicle – saline 0.9% + tween 80%), positive control (doxorubicin 3 mg/kg, via i.p., only two administration for it toxicity), THA (150 mg/Kg), THA 3-6 (50 mg/Kg) and TT2 (11) (50 mg/Kg). Day 7, animals were sacrificed and removed the fibers. Excess host tissue was removing and the fibers were transferred into six-well plate with prewarmed RPMI + FBS medium, were added MTT solution (1.25 mg/mL), and incubated during 4 h at 37°C, without light. Medium were aspirated and the fibers keeping in 2,5 % protamine solution (6 ml/well) for 24 h at 4°C. One fiber by well into the medium were cut in half and formazam crystal were dissolved in 250 µL de DMSO and incubated by 4 h, protecting of light. Aliquot of 150 µL were transferred to 96 well plates and were read by spectrophotometry at 550 nm. Results were expressed as (mean Odd7 -mean ODd0)/(mean ODd0))X100%) [25, 26].

RESULTS

Chromatographic analysis of TT2 (11)

The compound isolated from TT2 fraction (petroleum – ethyl acetate, 1:1), yielding 0.61 % (280 mg), is the major compound from this fraction and from methanol extract of stem of *Tabebuia hypoleuca* (TTM). It was an amorphous solid, RF= 0.77 with violet color on anisaldehyde, soluble in dichloromethane and ethyl acetate. This compound was 66.9% of purity. By spectral analysis (Mass Chromatography) it was a triterpene, isopropenil 5a,5b,8,8,11ª,13b-hexamethylcasahidro-9H-ciclopenta[a]crisen-9-one (A-neogommacer-22(29)-en-3one; hopenone b),$C_{30}H_{48}O$. In the chromatogram this compound has an Rt (retention time) = 47.64 min (66.9%). Other peaks in this chromatography profile were at Rt = 44.98 min (9.04%) and Rt= 42.98 min (6.46%), (Figure 1). By comparison of mass spectrum of peak Rt= 44.98 min with NITS -11 base date was possible identify this compound as lupenone (lu-20(29)-en- 3 one), with molecular ion at 424 m/z and ion base at 205. Peak at Rt= 42.98min was D- friedoolean-14-en-3-one (taraxerone, taraxeron), with molecular ion at 426 m/zand base ion at 204 (Figure 2 and 3).

In vitro antiproliferative assay

In the first *in vitro* antiproliferative evaluation using a triad human tumor cells (glioma,U251; breast, MCF-7 and renal, 786-0) (Table 1) of hexane, ethyl acetate, methanol extracts and fractions isolating from ethyl acetate and methanol extracts demonstrated the potency of some fractions with selectivity against tumor cell lines . For example TT2, fraction proceeding from methanol extract of *T. hypoleuca* stem had the more potent activity for MCF- 7 cell line (TGI= 8.5 ug/ml) and good activity for U252 cell line (TGI= 25.9 ug/ml). Some fractions separate from ethyl acetate extract of leaves were potent too (THA-3, THA-4, and THA-7). THA-4 had a TGI value of 14.3 ug/ml again renal human tumor cell.

The active samples above were assay by its *in vitro* antiproliferative activity against nine human tumor lines and one no tumor human line (Table 2). All sample were active. However, according to Fouche et al., (2008) the more potent were two fractions from ethyl acetate extract of leaves (THA -4 and THA- 7 on renal tumor cell line with TGI of 9.1 and 12.5 respectively) and THA- 4 on ovarian human tumor cell line OVCAR-3 with TGI of 10.6 ug/ml. The THA- 4 fraction has activity against leukemia, colon and prostate too.

A bioguide isolation described in table 3, was possible to demonstrate the promissory antiproliferative activity from THA (3-6) on glioma or melanoma cells and renal cells with TGI 11.6 and 17.91 ug/ml respectively. In this case, it showed a relative selectivity with no tumor cell line. However, the semi-pure major compound from TT2 fraction named TT2 (11) was not active. Thus, the antiproliferative activity from TT2 fraction against melanoma, breast and renal tumor cells was not associate to the compound isolating.

In vivo assays

Acute toxicity

No evidence of toxicity was observe 4h after administration of 50, and 100 mg/kg dose of ethyl acetate extract (THA) as well as during the following 14 days when the animals were kept under observation. All animals had weighting gain satisfactory and any difference on the relative weight of organs between control and treatment groups. Therefore, the above dosages were consider safe for the following experiments *and in vivo* animal tests were carry out.

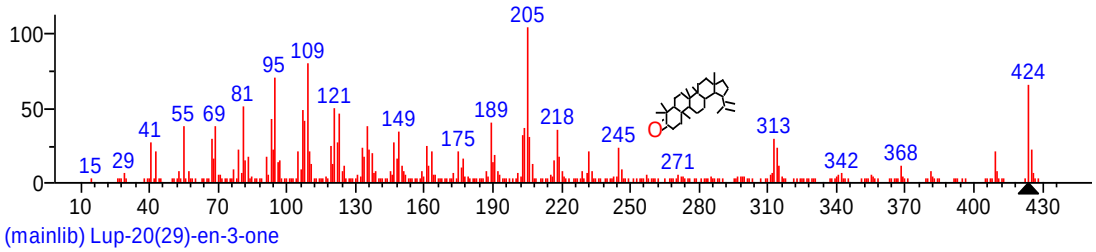


Figure 2 Mass spectrum and structure of lupenone

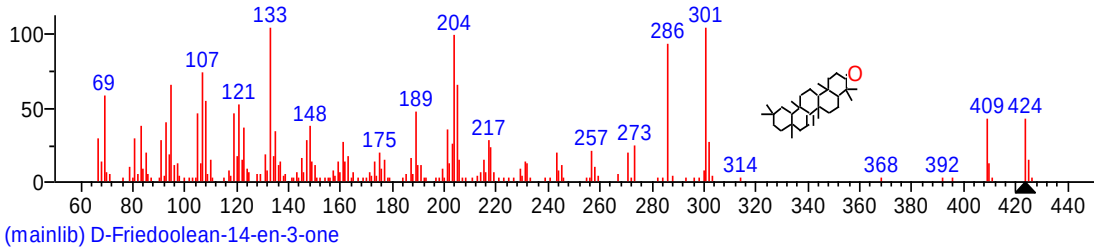


Figure 3 Mass spectrum and structure of taraxerone

Table 2 Antiproliferative activity of doxorubicin, some fractions and THH against nine human cancer cell lines and one no tumor cell

	TGI (ug/ml)										
	u	**m**	**a**	**7**	**4**	**p**	**o**	**h**	**k**	**media**	**q**
Doxorubicin	1,3	1,2	17,1	0,8	3,1	1,0	2,2	10,2	0,9	4,2	1,3
TT-2	37,0	202,5	211,2	51,1	156,5	50,8	61,3	74,6	13,2	95,4	121,1
THA-3	38,3	149,7	122,7	36,2	47,6	35,0	30,0	70,5	148,0	75,3	65,1
THA-4	69,5	50,5	>250	9,1	178,3	30,8	10,6	29,5	26,8	>72,8	75,0
THA-7	73,6	154,3	219,6	12,5	180,2	114,5	98,3	95,9	60,3	112,1	250
THA-8	>250	>250	>250	44,0	>250	206,5	>250	>250	>250	>222,3	>250
THM-6	119,4	113,0	96,4	62,1	182,5	85,5	63,1	88,5	>250	>117,8	>250
THH	139,4	163,7	96,47	62,1	182,5	93,0	57,3	222,0	145,7	129,1	51,8

Tumor cell lines: u = U251 (glioma, SNC), m = MCF-7 (breast); a =NCI-ADR/RES (ovarian phenotype resistance to multiple drugs); 7 = 786-0 (renal); 4 = NCI-H460 (lung, type small cells); p = PC-3 (prostate); o = OVCAR-3 (ovarian); h = HT29 (colorectal); k = K562 (leukemia).
No tumor line: q = HaCat (immortalized skin human keratocytes) TGI: Total Growth Inhibition

Table 3 Antiproliferative activity of doxorubicin, THA, some sub- fractions and semi- pure compound TT2 (11) against human cancer cell lines and one no tumor cell

	TGI (ug/ml)						
	2	M	4	7	H	K	**Cat**
Doxorubicin	<0,25	<0,25	<0,25	<0,25	0,60	<0,25	<0,25
THA	72,2	>250	>250	88,60	175,61	>250	153,33
THA- (3-6)	11,16	>250	>250	17,91	42,14	>250	33,12
TT2 (11)	>250	>250	>250	>250	>250	>250	>250
TT2 (13)	150,21	>250	>250	110,36	>250	>250	156,45
TT2 (19-20)	30,83	>250	>250	40,35	136,55	>250	85,75

Tumor cell lines: 2 = U251 (glioma, SNC), m = MCF-7 (breast); 7 = 786-0 (renal); 4 = NCI-H460 (lung, type small cells); h = HT29 (colorectal); k = K562 (leukemia).
No tumor line: Cat = HaCat (immortalized skin human keratocytes) TGI: Total Growth Inhibition.

Ehrlich solid tumor (footpad)

After the seventh day of the experiment, treatments with 50, 75, and 100 mg/kg of THA and 10 mg/kg of 5-Fluorouracil (5-FU) there was a tendency to inhibited the tumor growth for the treatment with 100 mg/Kg of THA. The decreasing of the tumor volume were significant for 5- FU and 100 mg/Kg THA after the first and second administration (for the first administration 23.87% of tumor inhibition for 5- FU and 62.3 % for THA 50 and 100 mg/Kg, respectively). After the second administration the tumor volume were inhibited 72.1% for 5-FU and 52% for 100 mg/Kg of THA (Fig. 4).

The tumor weight ending the study shown a decreasing of the tumor with the treatment of ethyl acetate extract at 100 mg/Kg but it was not significant, including the group treatment with 5- FU (Figure 5).

The toxicity signs evaluated in this experiment shown not toxic effect of the ethyl acetate extract. There was a good weight gain and there were no difference in the relative weight of the organ between control group and treatments groups (Figure 6).

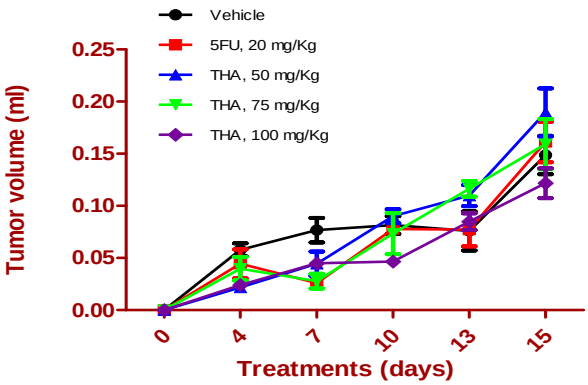
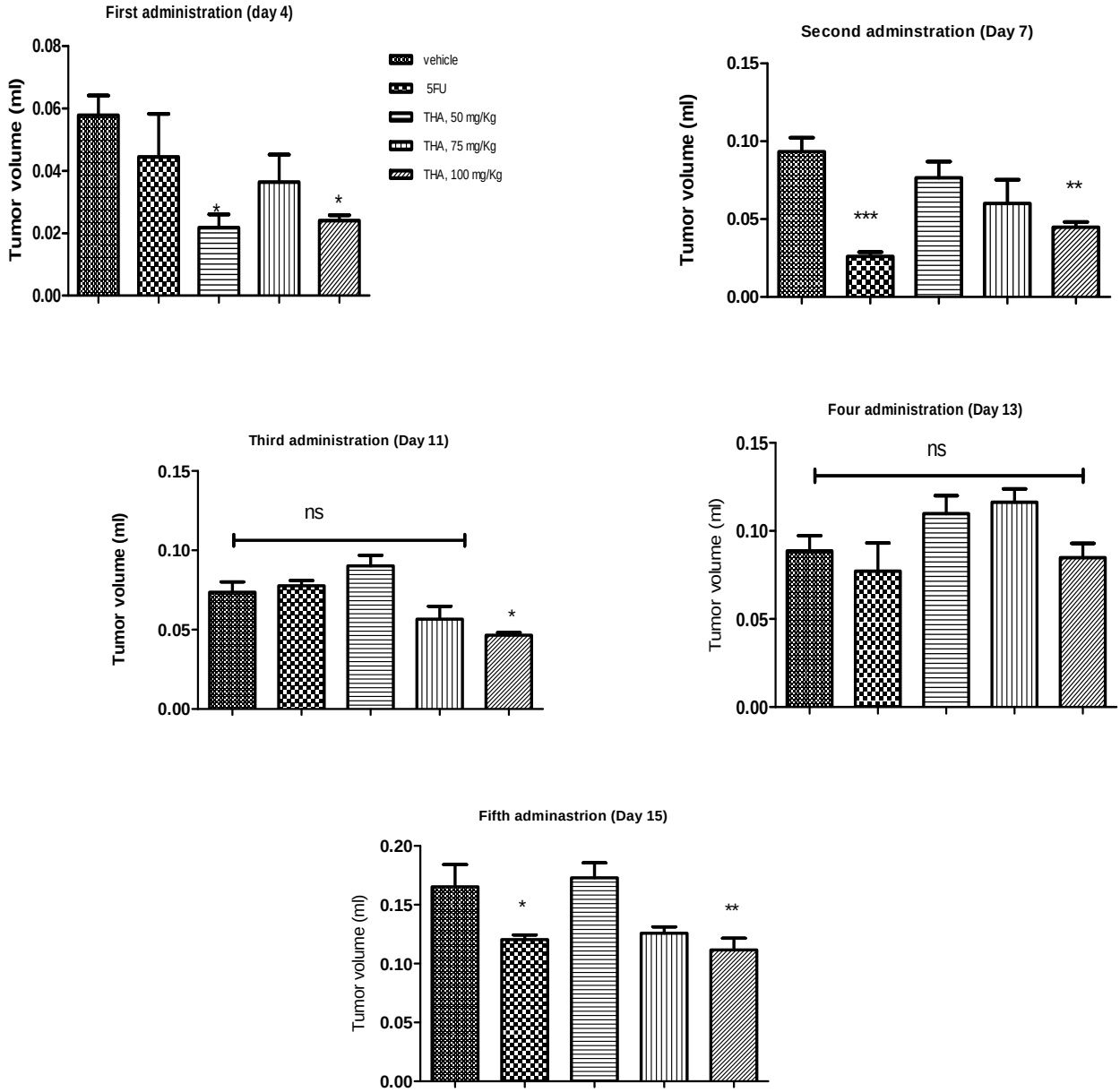


Figure 4 Tumor volume variation induced by Ehrlich cells in mouse hind footpad during the treatment with ethyl acetate extract from leaves of *Tabebuia hypoleuca*; * p < 0.05, Dunnet´s Multiple Comparison Test.



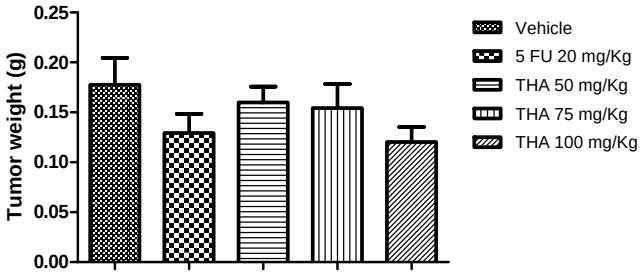


Figure 5 Tumor weight variation induced by Ehrlich cells in mouse hind footpad during the treatment with ethyl acetate extract from leaves of *Tabebuia hypoleuca.*

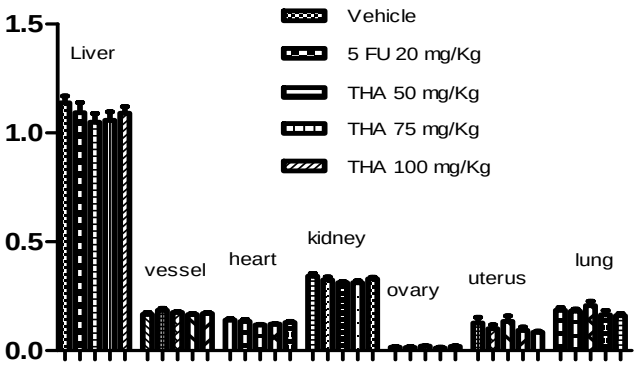


Figure 6 Relative organ weight of experimental groups treated with ethyl acetate extract (THA)

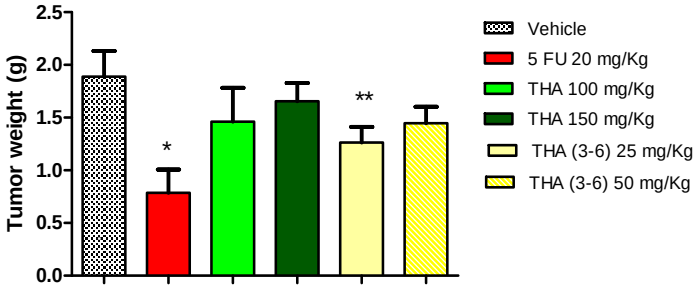


Figure 7 Tumor weight variation induced by Ehrlich cells in mouse back during the treatment with ethyl acetate extract and one fraction from leaves of *Tabebuia hypoleuca*

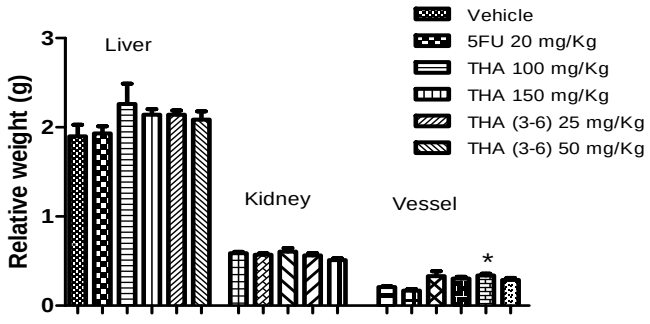


Figure 8 Relative organ weight of experimental groups treated with ethyl acetate extract (THA) and the fraction THA (3- 6).

Ehrlich solid tumor (back)

In this experiment the single tumor development in the back of mice decreased with all treatments. However, it was significant by 5- FU (positive group) and with the fraction THA (3- 6) in the doses of 25 mg/Kg respect to control group (Figure 7). This result confirm that the answer shown by ethyl acetate extract is associate that the presence of active compounds in the fraction THA (3- 6). Not toxic effect was shown with the treatment of the ethyl acetate extract and the fraction. There were no difference in the relative weight of the organ between control group and treatments groups (Figure 8).

Hollow fiber assays with MCF-7

On day seven, the fibers were removed to quantify cell proliferation. As shown in the Figure 9, the crude extract of ethyl acetate from leaves (THA) reduced cell proliferation of tumor cells MCF-7 (59.1%) at the doses of 150 mg/Kg/day, the treatment with the semipure compounds THA (3-6) reduced at 43% and doxorubicin reduced cell proliferation of tumor (60.6%) and optical density at doses of 3 mg/kg, only with two administration. The treatment with TT2 (11) have not activity on this cell proliferation at the doses of 50 mg/Kg/day.

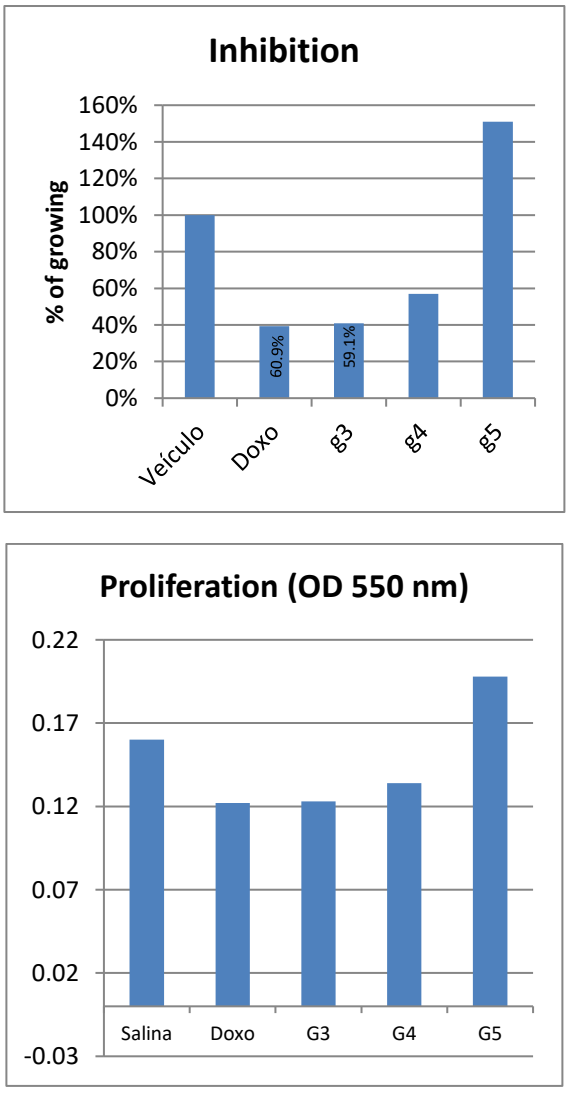


Figure 9 Effect of ethyl acetate crude extract (g3) and semipure compounds (g4 and g5) from *T. hypoleuca* on the cell line MCF-7 growing assessed by the *in vivo* hollow fiber assay

The hollow fibers were quite well tolerated by the animals, andno signals of rejection were detected. The treatments with the crude extract and semipure compounds obtained from *T. hypoleuca* and doxorubicin did not affect the conditions of the mice beyond acceptable limits, and no deaths were noticed (Table 4). No significant changes in bodyweight were seen in the crude extract- or semi pure compounds-treated groups. Moreover, in order to evaluate the toxicological aspects related to drug treatment, histological analyses of the liver, kidneys, heart, lungs, and spleen of the treated animals were performed; however, the treatments with the crude extract and semipure compounds from *T. hypoleuca* did not affect the morphology of the analyzed organs.

Table 4 Antitumor efficacy of the crude extract and semipure compounds obtained from *T. hypoleuca* assessed by the in vivo hollow fiber assay

Treatment	Dose (mg/kg/day)	Survival	Proliferation (OD 550 nm)	Inhibition (%)
Vehicle	-	5/5	0.163	-
Doxorubicin	3	5/5	0.122	60.61
Ethyl acetate extract of leave	150	5/5	0.123	59.15
THA (3-6)	50	5/5	0.134	43
TT2 (11)	50	5/5	0.198	-

Data are presented as mean for 5 animals; starting one day after hollow fiber implantation, the animals were treated for six consecutive days via the intraperitoneal route. The negative control was treated with the vehicle used for diluting the test substance (saline 0.9% + tween 80%). Doxorubicin was used as a positive control.

DISCUSSION

Over the past few decades, cancer has turned out to be the second most frequent cause of death around the world and a major concern for pharmaceutical industries. The discovery of new drugs derived from medicinal plants still plays an important role in cancer chemotherapy, despite the development of synthetic drugs (Cragg and Newman, 2009).

Most of the commonly used cytotoxic anticancer drugs were discovered through random high-throughput screening of synthetic compounds and natural products in cell-based cytotoxicity assays. Despite the number and chemical diversity of these agents, the mechanisms of action are limited, and most compounds are DNA-damaging agents with a low therapeutic index. With this screening approach, mechanism of action is not a primary determinant in selecting agents for further development, and, as a result, none of the current drugs directly targets the molecular lesions responsible for malignant transformation. The initial National Cancer Institute (NCI) high-throughput screen used the highly chemosensitive P388 leukemia cell line, but this screen failed to identify drugs that were active against the common adult solid tumors. In the mid-1980s, the NCI implemented a new in vitro disease-oriented screen consisting of 60 human tumor cell lines representing nine common forms of cancer. It remains to be determined whether selective activity *in vitro* against cell lines representing a particular histologic form of cancer will be predictive for antitumor activity *in vivo* (Balis, 2002).

Tabebuia spp. have been reported with some pharmacological activities in different part of the world. For example, *Tabebuia roseae* ethanolic leaf extract is said to have remarkable antimicrobial activity against a wide range of gram positive and gram negative bacteria. The essential oil of *Tabebuia rosea* leaf and bark is reported to be cytotoxic which may be due tothe presence of o-xylene, 2,4-dimethylhexane, methyl cyclohexane (53.13%), methylbenzene, 3-Pentene-2-one (0.11%) and, the alkaloid extract from *Tabebuia rosea* leaves is preferentially

said to be cytotoxic to human T-cell leukemia (MOLT-4) cells in a dose and time dependent manner with the absence of genotoxicity (Ramalakshmi and Muthuchelian, 2011).

Hemamalini et al., 2012 reported *in-vivo* anticancer activity of *Tabebuia rosea* (Bertol) DC. leaves on Dalton's ascetic lymphoma in mice and it antioxidant activity on lipid peroxidation, glutathione, superoxide dismutase, and catalase.

Tabebuia argentea produce anticancer agent, lapachol and it is ability to interfere with the bioactivities of enzymes known as, topoisomerases, a group of enzymes that are critical for DNA replication in cells. The antitumor activity of lapachol may be due to its interaction with nucleic acids and the interaction of the naphthaquinone moiety between base pairs of the DNA helix. Lapachol has other activities as antimetastatic activity, antimicrobial and antifungal activity, antiviral activity, anti-inflammatory, antiparasitic activity, leishmanicidal activity and molluscicidal activity (Channabasava and Melappa, 2014).

The flower of Tabebuia rosea has anti-cancer activity against human liver cancer (Solomon et al., 2015). The chemical composition of *Tabebuia spp*. has been extensively studied and a variety of constituents have been isolated, such as furano naphthoquinones, naphthaquinones, quinones, benzoic acids, cyclopentene dialdehydes, iridoids, and phenolic glycosides. Its biological properties have been related mainly with the presence of naphthaquinones, which constitute the most prevalentactive chemical group in some Tabebuia spp. Among the naphthaquinones, lapachol and β-lapachone are the two compounds that attracted the highest interest. Lapachol presents potent antiproliferative properties against various tumor cell, nonetheless, a phase I clinical trial was prematurely interrupted, due to the observance of secondary effects such as nausea and vomiting (Castellanos et al., 2009; Li et al., 1995; Block et al., 1974 and Pires et al., 2015).

A study on molecular biology to Bignoniaceae family confirm that is taxon is chemical characterized by terpenoids (NO = 319), quinones (NO = 151), not nitrogen derivate of large chain from acetic acid via (NO = 37), alkaloids (NO = 38), special aromatic derivate (NO = 194) and flavonoides (NO = 105), between others compounds as lignans, flavonoids, cinnamic and benzoic acids (Cipriani et al., 2012; Duarte et al., 2014). However, *Tabebuia spp*. had not reported the presence of pentacyclic triterpenes as the major chemical compound. For this reason, it is the first report of the presence of pentacyclic triterpenes in the genera. Previous study with some endemic and native Cuban plants showed the preliminary cytotoxic effect from *Tabebuia hypoleuca* by *Artemia salina´*s Test and the presence of triterpenes as major compound in this specie (Sánchez et al., 2017).

In our study, extract and fractions from *Tabebuia hypoleuca* show cytotoxicity or *in vitro* antiproliferative activity against some tumor cells with different selectivity. For example, TT2 a fraction isolated from total methanol extract of stem was very active against MCF-7, U252 and Khuman tumor cells. However, after separate the major compound in this fraction (TT2- 11) the activity decrease weakly for it compound. This compound was 69 % of purity by hopenone –b and other minor compounds as lupenone and taraxerone.

Lupenone have anticancer activity particularly activating apoptosis and inhibition of new vessel formation which halts the growth of cancer cell. Lupenone turned out to induce significant cytotoxicity against lymphoma and melanoma cells (Rakeshet al., 2017). Lupenone express activity against β-site amyloid precursor protein cleaving enzyme 1 (BACE1), which serve as a rate limiting step in amyloid beta (Aβ) production altering the course of Alzheimer's disease (Koirala et al., 2017).

Samarakkon et al., 2016 reported the cytotoxic activity against MCF-7 (estrogen receptor positive breast cancer), HepG2 (hepatocellular carcinoma) and AN3CA (endometrial cancer) from hopenone I, a pentacyclic triterpene isolated from the hexane extract of leaves of the mangrove plant *Scyphiphora hydrophyllacea* C.F.Gaertn. Hopenone I has a similar basic structure at hopenone b; with EIMS m/z 424 $[M]^+$(23), 409 (18), 381 (100), 202 (12), 188 (14), 175 (16), 161 (36), 148 (17), 133 (27), 119 (19). However, hopenone I has an unsaturation on the pentacyclic ring different to hopenone b and the side chain present two methyl group possibility associate to the biological activity from this compound.

Quan, 2008 isolated Hopenone-B(1), hopenol-B(2),22-Hydroxyhopan-3-one (3), erythrodiol 3-palmitate(4)and 5β,24-cyclofriedelan-3-one(5) from *Pericampylus glaucus.* Among them, only the compound 4 inhibited the proliferation of K562 cells with the inhibition rate of 47% at 100μg/mL, however, hopenone b is not active.

Ma et al., 2016 described the inhibition cancer cell growth by inducing apoptosis in non-small cell lung cancer cells by taraxerone triterpene (IC50, 75 uM). Mo et al., 2012 reported the antioxidant activity by this compound isolated from *Sedum sarmentosum,* with inhibitory effect on nitric oxide generation was significantly more effective than that of caffeic acid and/or gallic acid.

This finding appoint that the *in vitro* antiproliferative activity is a combination of all compounds in this fraction (TT2) and it is not by the major compound, hopenone- b.

Extracts and fractions for the leaves from *Tabebuia hypoleuca* are more active than stem´s fractions on *in vitro* and *in vivo* antiproliferative assays. THA-4, THA-7, THA (3-6) were active on kidney, ovary, colon, prostate and leukemia human tumor cells. Any extract or fraction showed enough selectivity against no human cell line.

Ethyl acetate extract (THA) shown a tendency to decrease of tumor growth and tumor volume in doses- depended with best result by 100 mg/Kg on Ehrlich solid tumor with mouse mammary adenocarcinoma (footpad) and this effect was confirmed on Ehrlich solid tumor on the back in mice. In this second assay, the semipure compound THA (3-6) isolated from THA shown a significant decrease on the tumor weight. These finding indicate the necessary *in vivo* study with higher doses from THA and with THA (3-6) on Ehrlich solid or ascitic tumor and in other xenograft tests with human tumor cells as renal tumor line.

The hollow fibers were quite well tolerated by the animals, and no signals of rejection were detected. Hollow fiber test using MCF-7 (human breast tumor cell) was a good model by THA at 150 mg/Kg dosed, THA (3-6) and TT2 (11). However, TT2 (11) was not active in this cell line. For this reason we recommend to test TT2 on Hollow fiber with MCF-7 and 5-Fluorouracil (5-FU) as a positive control to verified *in vitro* answer of the total fraction on this tumor cell and isolated other chemicals from this fraction to justify the *in vitro* antiproliferative activity. In the case of THA and THA (3- 6) from leaf, the effect would be associate at the presence of other pentacyclic triterpenes. Se recommend to identify these compounds and to repeat the assay with other human tumor cell as kidney, colon, ovary where these extract and semipure compounds were more active on the *in vitro* antiproliferative test. Doxorubicin had not a good action in this animal assay. Doxorubicin is an anthracycline commonly used to treat several solid tumors, acute leukemia and malignant lymphoma (Elbialy and Mady, 2015). Any toxic effects were shown on *in vivo* tests.

The Ehrlich tumor is primarily a mouse mammary adenocarcinoma in females (Paul Ehrlich, 1906) and, even undifferentiated, it is possible suffer the influences of anti-inflammatory drugs in genesis of mammary

cancer (Souza et al., 2015; Vendramini et al., 2010). Ehrlich tumor is a rapidly growing carcinoma with very aggressive behavior (Segura et al., 2000). It is able to grow in almost all mice strains which suggest that the recognition and immune responses to this tumor are independent of MHC. This characteristic suggests that the controlling of Ehrlich tumor is related more with innate immunity, specially the inflammatory response, than with T-cell responses (Iwamoto et al., 2015).

Solid tumors are structures resembling organs in their complexity and heterogeneity. Inside these tumors there are differences in pH, oxygen pressure, and nutrient flux, which often contribute to tumor resistance to chemotherapy due to irregular drugs distribution inside the tumor matrix. Therefore, the development of experimental models to complement *in vitro* drug screening is necessary due to the limitations inherent to cell cultures to predict the behavior of solid tumors to chemotherapy (O. Tr´edan et al., 2007).

Hollow fiber is a standard *in vivo* model that allows simultaneous evaluation of multiple cell lines. It was developed by Hollingshead et al. 1995 as a model with a shorter evaluation time and a reduced compound requirement compared to traditional xenograft models (Temmink et al., 2007 and Bezerra et al., 2015).

Based on the results presented here, we conclude that *Tabebuia hypoleuca* has promising antitumor activity, without side effects. Further studies will be performed to clarify the therapeutically doses by in vivo models and the biochemical pathways involved in this activity. These results highlight the importance of *Tabebuia hypoleuca*as a potential source of compounds against cancer.

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Key words

Tabebuia hypoleuca, *in vitro* antiproliferative, Ehrlich solid tumor, Hollow fiber, MCF-7, hopenone b

Abbreviations

TT2: Major fraction from stems of Tabebuia hypoleuca
THA: T. hypoleuca leaves ethyl acetate crude extract
TH 3-6: semi pure compounds from leaves
TT2 -11: semi pure compound from stem
THH: hexane crude
THA: ethyl acetate crude
THM: methanol crude
TLC: thin-layer chromatography
RPMI 1640: Medium
FBS: fetal bovine serum (FBS,
DMSO: dimethyl sulphoxide
UACC-62: melanoma
MCF7: breast
786–0: kidney
NCI-H460: lung
PC-3: prostate
OVCAR-3: ovary
HT29: colon
K-562: leukemia
HaCat: no tumor line, immortal keratinocyte from human skin
TGI: Total growth inhibition
GI50: Medium growth inhibition
5- FU: 5-fluorouracil
Rt: retention time

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